

# Secretory Protein Profiling Reveals TNF- $\alpha$ Inactivation by Selective and Promiscuous Sec61 Modulators

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## SUMMARY

Cotransins are cyclic heptadepsipeptides that bind the Sec61 translocon to inhibit cotranslational translocation of a subset of secreted and type I transmembrane proteins. The few known cotransin-sensitive substrates are all targeted to the translocon by a cleavable signal sequence, previously shown to be a critical determinant of cotransin sensitivity. By profiling two cotransin variants against a panel of secreted and transmembrane proteins, we demonstrate that cotransin side-chain differences profoundly affect substrate selectivity. Among the most sensitive substrates we identified is the proinflammatory cytokine tumor necrosis factor alpha (TNF- $\alpha$ ). Like all type II transmembrane proteins, TNF- $\alpha$  is targeted to the translocon by its membrane-spanning domain, indicating that a cleavable signal sequence is not strictly required for cotransin sensitivity. Our results thus reveal an unanticipated breadth of translocon substrates whose expression is inhibited by Sec61 modulators.

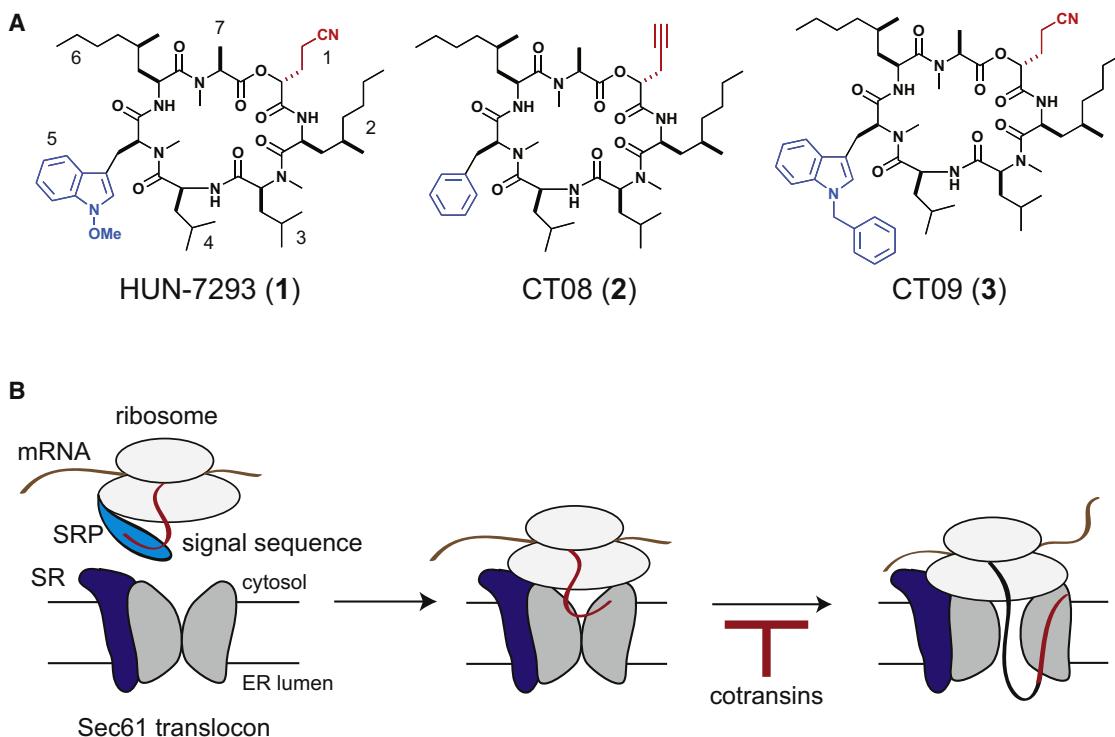
## INTRODUCTION

Cotranslational translocation into the endoplasmic reticulum (ER) is an essential, early step in the biogenesis of secreted and transmembrane proteins. Selective delivery of these proteins to the ER is initiated when the first hydrophobic domain, either a cleavable N-terminal signal sequence or an internal transmembrane domain, emerges from the ribosome. This hydrophobic domain is initially captured by the signal recognition particle (SRP), which targets the ribosome-nascent chain complex to the ER membrane via the SRP receptor (Egea et al., 2005; Halic and Beckmann, 2005). The nascent chain is then

transferred to the Sec61 translocon, a multisubunit transmembrane protein complex that mediates cotranslational translocation and membrane integration of nearly all secreted and transmembrane proteins (Rapoport, 2007; Mandon, et al., 2009). Once at the translocon, the signal sequence or transmembrane domain is thought to trigger a conformational change in Sec61 $\alpha$ , which opens the translocation channel for polypeptide entry into the ER lumen and/or the lipid bilayer (Hegde and Kang, 2008; Plath et al., 1998).

We (Garrison et al., 2005) and another group (Besemer et al., 2005) previously characterized the biological mechanism of "cotransins," a class of cyclic heptadepsipeptides structurally related to the fungal natural product HUN-7293 (compound 1, Figure 1A). Cotransins potently inhibit the cotranslational translocation of a subset of secreted and transmembrane proteins, resulting in their proteasomal degradation in the cytosol. Mechanistic studies with VCAM (vascular cell adhesion molecule-1) suggested that cotransins inhibit gating of the Sec61 translocon by the VCAM signal sequence, thereby preventing the nascent polypeptide from accessing the ER lumen (Figure 1B). Consistent with this model, a clickable photoaffinity probe related to compound 2 (CT08, Figure 1A) identified the core translocon subunit, Sec61 $\alpha$ , as a direct high-affinity target (MacKinnon et al., 2007). Moreover, signal sequence swapping experiments revealed that cotransin sensitivity is determined by the precise identity of the N-terminal signal sequence (Garrison et al., 2005; Besemer et al., 2005). Thus, cotransins appear to selectively disrupt the decisive interaction between Sec61 $\alpha$  and a subset of cleavable N-terminal signal sequences.

Given that many proteins with cleavable signal sequences are attractive therapeutic targets, understanding the basis of cotransin selectivity is of considerable interest. To date, only five cotransin-sensitive proteins (VCAM, ICAM-1, E-selectin, P-selectin, and VEGF-A) have been identified, all of which contain cleavable signal sequences lacking any obvious sequence similarity (Garrison et al., 2005; Harant et al., 2007; Boger et al., 1999). Mutagenesis studies have suggested a rough correlation between signal sequence hydrophobicity and cotransin

**Figure 1. Cotransins, Cyclic Depsipeptide Inhibitors of Cotranslational Translocation**

(A) Chemical structures of HUN-7293, CT08, and CT09.

(B) Cotransins inhibit cotranslational translocation of a subset of secreted and transmembrane proteins by preventing signal sequence-dependent opening of the Sec61 translocon. Signal recognition particle (SRP); signal recognition particle receptor (SR).

sensitivity, although numerous exceptions to this trend imply a more complex relationship (Harant et al., 2006, 2007). Similarly, little is known about the relationship between cotransin structural elements (e.g., its amino and hydroxy acid side chains) and substrate selectivity/promiscuity. An extensive analysis of HUN-7293 variants revealed that side-chain or backbone modifications can dramatically affect potency (Chen et al., 2002). However, the analysis of only two secretory protein substrates in this study (VCAM and intercellular adhesion molecule-1, ICAM-1) left the issue of selectivity largely unresolved. We therefore sought to define a broader range of cotransin-sensitive secretory and transmembrane substrates, and at the same time, determine whether substrate selectivity can be altered by side-chain modifications.

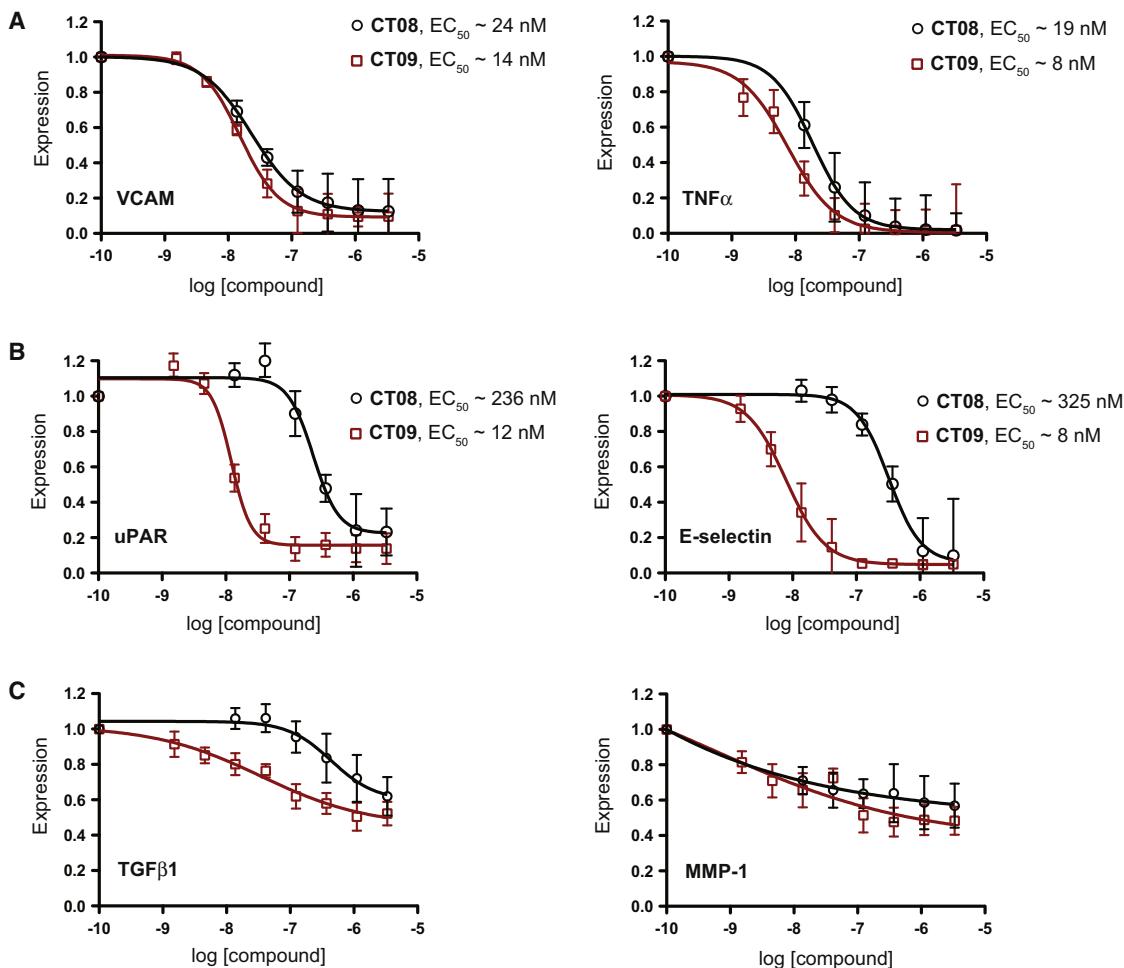
## RESULTS AND DISCUSSION

In the structure-activity study described above (Chen et al., 2002), most of the HUN-7293 variants were at least 20 times more potent at blocking the expression of VCAM, as compared with ICAM. We were intrigued by the sole exception, a compound we have named CT09 (compound 3, Figure 1A), which had the opposite preference, displaying slightly increased potency against ICAM relative to VCAM. CT09 differs structurally from HUN-7293 by the presence of an *N*-benzyl indole, which replaces the *N*-methoxy indole at position 5 (Figure 1A). Although only two secretory proteins were examined, the results of Chen

et al. led us to hypothesize that subtle side-chain differences could significantly impact cotransins' selectivity/promiscuity.

We therefore synthesized CT09 and quantified its effects on the expression of 25 secreted and transmembrane proteins implicated in autoimmune disease, inflammation, and cancer (see Table S1 available online). In parallel, we profiled the related cyclic heptadepsipeptide CT08 (compound 2, Figure 1A), which we previously found to inhibit VCAM expression at low nanomolar concentrations (MacKinnon et al., 2007). We quantified endogenously expressed proteins in primary human cells (endothelial, peripheral blood mononuclear, and bronchial epithelial cells) under stimulatory conditions designed to model human pathophysiology (Plavec et al., 2004; Berg et al., 2010). Protein expression levels were quantified by validated immunoassays (for details, see Supplemental Experimental Procedures).

Strikingly, CT08 and CT09 were equipotent at blocking the expression of a subset of proteins but had vastly different potencies toward the majority of proteins tested. Based on their relative sensitivities to CT08 and CT09, the 25 proteins on the panel can be divided into three classes (for the complete data set, see Figure S1). The first class comprises only two proteins, VCAM and TNF- $\alpha$ , both of which were sensitive to low nanomolar concentrations of CT08 or CT09 (Figure 2A). In the second and largest class (72% of the panel) are proteins whose expression was inhibited by CT09 much more potently than CT08 (Figure 2B; Figure S1B). This class is exemplified by the urokinase plasminogen activator receptor, uPAR (20-fold more sensitive to CT09



**Figure 2. CT08 and CT09 Differentially Inhibit Secretory and Transmembrane Protein Expression**

Primary human cells were stimulated under various proinflammatory conditions (see [Supplemental Experimental Procedures](#) for details) in the presence of increasing concentrations of CT08 or CT09. After 24 hr, expression levels of a total of 25 secreted and membrane proteins (see [Table S1](#) for details) were quantified by cellular immunoassays and normalized to DMSO controls (mean  $\pm$  SEM;  $n = 3$ ). EC<sub>50</sub> values were estimated by curve fitting with Prism software (for the complete set of dose-response curves and 95% confidence intervals, along with EC<sub>50</sub> values grouped by system, see [Figure S1](#)).

(A) Class I proteins, VCAM and TNF- $\alpha$ , are similarly sensitive to CT08 and CT09.

(B) Class II proteins, uPAR and E-selectin, show greater sensitivity to CT09 than CT08.

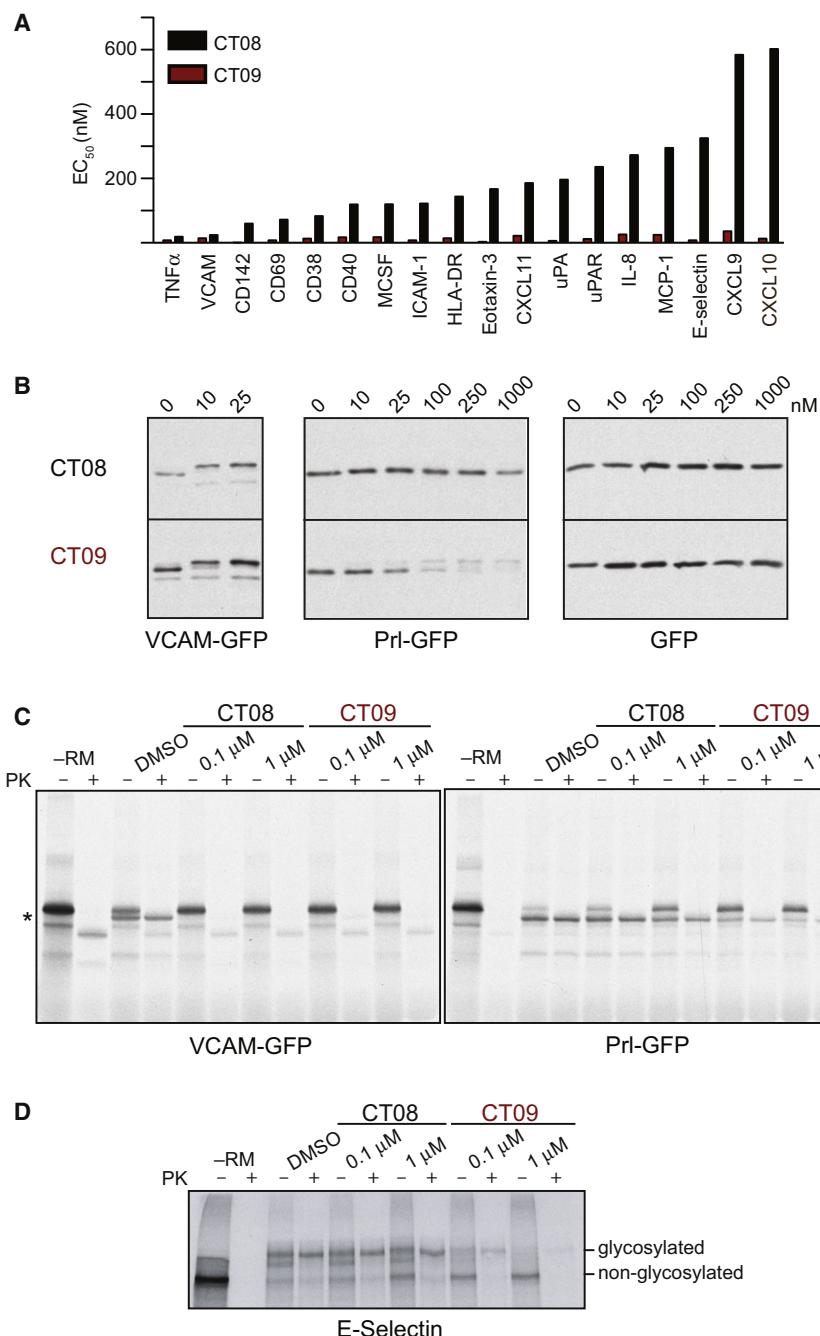
(C) Class III proteins, TGF- $\beta$ 1 and MMP-1, show <60% inhibition by 3.3  $\mu$ M CT09 or CT08.

and the cell adhesion protein, E-selectin (40-fold more sensitive to CT09). Vascular endothelial growth factor receptor-2 (VEGFR2) provides an extreme example of this selectivity, as it was unaffected by up to 3.3  $\mu$ M CT08, yet was maximally inhibited by approximately 100 nM CT09 (>80-fold more sensitive to CT09; [Figure S1B](#)). Finally, a third class of proteins (20% of the panel) was only partially affected by micromolar concentrations of either CT08 or CT09 (<60% inhibition at 3.3  $\mu$ M) ([Figure 2C](#); [Figure S1C](#)).

A comparison of CT08 and CT09 EC<sub>50</sub> values across the panel reveals two salient features ([Figure 3A](#)). First, CT09 is extremely potent, inhibiting the expression of most secreted and transmembrane proteins at concentrations below 100 nM. By contrast, at a concentration of 100 nM, CT08 completely inhibited the expression of only two proteins VCAM and TNF- $\alpha$  ([Figure 2A](#)); higher concentrations were required to inhibit the expression of

most other proteins ([Figure 2B](#); [Figure S1B](#)). Second, the data reveal an unconventional structure-activity relationship: side-chain differences between CT08 and CT09 ([Figure 1B](#)) impart dramatic potency differences (EC<sub>50</sub> ratios) that vary over two orders of magnitude depending on which secretory protein is examined (exemplary CT08:CT09 EC<sub>50</sub> ratios: approximately 1.7 for VCAM; approximately 20 for uPAR; approximately 200 for CXCL10; [Figure 1B](#)). Moreover, the rank order of protein sensitivity (ranked by increasing EC<sub>50</sub>) differs between CT08 and CT09. For example, whereas TNF- $\alpha$  is the most CT08-sensitive protein ([Figure 3A](#)), there are nine proteins that are equally or more CT09 sensitive than TNF- $\alpha$  ([Figure S1B](#)).

We hypothesized that the differential selectivity with which CT08 and CT09 inhibit secretory protein expression arises at the level of cotranslational translocation and that such differences depend primarily on the signal sequences that mediate



**Figure 3. CT08 Is Relatively Selective, whereas CT09 Is a Highly Promiscuous Inhibitor**

(A) EC<sub>50</sub> values for CT08 and CT09 across the panel of 25 proteins were estimated by curve fitting with Prism. Proteins are listed in order of decreasing sensitivity to CT08.

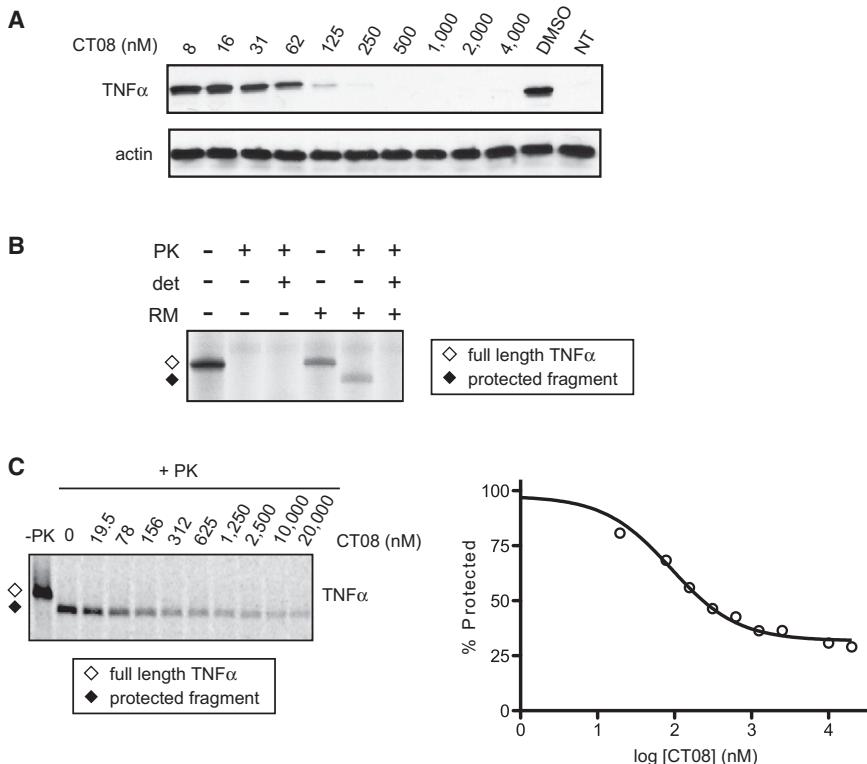
(B) HeLa cells were transfected with plasmids encoding GFP or GFP containing the signal sequence (plus 10 mature domain residues) from VCAM or Prl fused to the N terminus. Transfected cells were treated with the indicated concentrations of CT08 or CT09 for 20 hr prior to cell lysis and western blot analysis with antibodies to GFP. (C and D) mRNA encoding the indicated proteins was translated in reticulocyte lysate in the presence of [<sup>35</sup>S]-Met, rough ER microsomes (RM), and the indicated concentrations of CT08 or CT09. The translated material was left untreated or treated with proteinase K (PK) and analyzed by SDS-PAGE followed by autoradiography. Asterisk indicates translocated VCAM/PrI-GFP.

was potently inhibited by CT09 (EC<sub>50</sub> approximately 25 nM, Figure 3B). The apparent reduced stability of full-length Prl-GFP relative to VCAM-GFP likely derives from the greater hydrophobicity of the Prl signal sequence, recently shown to be an important determinant in the degradation of secretory proteins mislocalized to the cytosol (Hessa et al., 2011). GFP lacking a signal sequence was unaffected by either CT08 or CT09. We confirmed the selectivity of CT08 versus CT09 by comparing their effects in an in vitro translocation system containing reticulocyte lysates and pancreatic ER microsomes. Consistent with the cell-based experiments, in vitro translocation of VCAM-GFP was abolished by low concentrations of both CT08 and CT09, whereas Prl-GFP was significantly more sensitive to CT09 than CT08 (Figure 3C). These experiments, coupled with the cell-based experiments in Figure 3B (VCAM-GFP and GFP constructs), also demonstrate that neither CT08 nor CT09 affects translation. Finally, in vitro translocation assays confirmed that full-length E-selectin is least 10-fold more sensitive to CT09 (EC<sub>50</sub> < 100 nM) than CT08 (Figure 3D), consistent with the cell profiling experiments. We conclude that CT09 broadly inhibits cotranslational translocation and is relatively indiscriminate with respect to signal sequence. By contrast, sensitivity to CT08 is highly signal sequence dependent.

We further characterized TNF- $\alpha$  because of its therapeutic relevance to autoimmune disorders (Palladino et al., 2003) and its potent inhibition by the more selective cotranslational translocation modulator CT08. It was particularly important to corroborate TNF- $\alpha$  inhibition in

translocon targeting. To test this hypothesis, we generated constructs containing the signal sequence plus ten mature domain residues of either VCAM or preprolactin (Prl) fused to the N terminus of GFP; both constructs also contained a C-terminal ER-retention signal (Snapp et al., 2006). Treatment of transfected HeLa cells with nanomolar concentrations of either CT08 or CT09 resulted in an upward shift in the apparent mobility of VCAM-GFP (Figure 3B), consistent with a lack of signal sequence cleavage due to inhibition of cotranslational translocation. By contrast, expression of the mature, signal-cleaved form of Prl-GFP was relatively insensitive to CT08 (EC<sub>50</sub> > 1  $\mu$ M), yet

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**Figure 4. CT08 Inhibits Cotranslational Translocation of TNF- $\alpha$ , a Type II Transmembrane Protein that Lacks a Cleavable Signal Sequence**

(A) Expression of TNF- $\alpha$  in COS-7 cells treated with CT08. Cell lysates were analyzed by western blotting. NT, not transfected.

(B) mRNA encoding TNF- $\alpha$  was translated in the presence of [ $^{35}$ S]-cysteine and rough ER microsomes (RM). The translated material was left untreated or treated with proteinase K (PK) in the presence or absence of detergent (det, Triton X-100). Samples were separated by SDS-PAGE and analyzed by autoradiography. The positions of full-length TNF- $\alpha$  (open diamond) and its protease-protected fragment (solid diamond) are indicated.

(C) In vitro translation/translocation of TNF- $\alpha$ , as in Figure 4B. Samples translated in the presence of increasing concentrations of CT08 were digested with proteinase K (+PK), separated by SDS-PAGE, and analyzed by autoradiography and phosphor-imaging. A dose-response curve was derived by quantifying the PK-protected TNF- $\alpha$  fragment.

multiple systems, as it represents the first example of a cotranslational substrate that lacks a cleavable signal sequence. TNF- $\alpha$  is cotranslationally inserted into the ER membrane with a type II orientation prior to its delivery to the plasma membrane (Utsumi et al., 1995; Kriegler et al., 1988); it has a cytoplasmic N-terminal tail, followed by a transmembrane-spanning region and an extracellular C-terminal domain that acts as a proinflammatory cytokine when liberated by extracellular proteases. In lieu of a cleavable signal sequence, the hydrophobic membrane-spanning domain serves as a “signal anchor” that mediates SRP-dependent targeting, engagement of the Sec61 translocon, and insertion into the lipid bilayer. As such, it is one of four proteins on our panel that relies on an internal signal anchor for cotranslational translocation (the other type II transmembrane proteins, HLA-DR, CD38 and CD69, were affected by higher concentrations of CT08, Figure 3; Figure S1B).

Because the inhibitory effects of CT08 and CT09 were revealed in a complex cellular system (peripheral blood mononuclear cells stimulated with LPS) in which endogenous TNF- $\alpha$  expression is regulated transcriptionally (Collart et al., 1990), posttranscriptionally (Wang et al., 1997; Kontoyiannis et al., 1999), and translationally (Han et al., 1990), we performed experiments to rule out indirect effects and test whether CT08 blocks TNF- $\alpha$  at the level of cotranslational translocation. We focused on CT08, due to its greater selectivity across the panel and thus lower likelihood of inhibiting TNF- $\alpha$  expression by indirect mechanisms.

We first expressed TNF- $\alpha$  from a cytomegalovirus promoter-driven plasmid in transiently transfected COS-7 cells. In this system, CT08 potently inhibited TNF- $\alpha$  expression with an EC<sub>50</sub> of less than 60 nM (Figure 4A). As observed with endogenously expressed proteins in primary human cells, the inhibitory effect

of CT08 toward ectopically expressed TNF- $\alpha$  was similar to its effect on VCAM expressed under identical conditions (MacKinnon et al., 2007). To confirm that

CT08 blocks TNF- $\alpha$  expression at the level of cotranslational translocation, we moved to the in vitro translocation system. Cotranslational insertion of TNF- $\alpha$  into the microsomal membranes was indicated by the formation of a protease-protected fragment consisting of its transmembrane and luminal domains. In the absence of microsomes, or when microsomes were disrupted by detergent, TNF- $\alpha$  was completely digested by added protease (Figure 4B). Consistent with the results obtained in transfected cells, cotranslational translocation of wild-type TNF- $\alpha$  into ER microsomes was inhibited by nanomolar concentrations of CT08 (Figure 4C).

The TNF- $\alpha$  validation experiments conclusively demonstrate that noncleavable signal anchors (found in all type II transmembrane proteins and many multispanning membrane proteins) can be as susceptible as cleavable signal sequences to cotranslational-mediated inhibition. This was unexpected because (1) previous studies indicated that increasing the hydrophobicity of signal sequences leads to decreased cotranslational sensitivity (Harant et al., 2006, 2007), and (2) signal anchors, by virtue of their requirement to stably span the lipid bilayer, are universally more hydrophobic than cleavable signal sequences (Martoglio and Dobberstein, 1998; Sakaguchi, et al., 1992). Thus, cotranslational sensitivity is not likely based on a single biophysical parameter such as signal sequence/anchor hydrophobicity, raising the possibility that more finely tuned cotranslational variants with differing substrate selectivity may be discovered.

## SIGNIFICANCE

Prior to this work, cotranslational cyclodepsipeptides had been shown to inhibit the expression of only five secreted

**and transmembrane proteins, all with cleavable signal sequences lacking any obvious sequence similarity. We made the surprising discovery that structural differences among cotransins (CT08 versus CT09) can drastically alter the range of inhibited proteins: CT09 inhibited the expression of 20 out of 25 proteins tested at low nanomolar concentrations, whereas CT08 exhibited selectivity for VCAM and TNF- $\alpha$ , affecting most of the remaining proteins only at higher concentrations. In the future, it may be possible to refine structure-activity relationships of CT08-like molecules such that even greater selectivity toward TNF- $\alpha$  is achieved. Such compounds may provide early leads for small-molecule anti-inflammatory agents that block the functional expression of TNF- $\alpha$ .**

## EXPERIMENTAL PROCEDURES

### Chemical Synthesis of Cotransins

CT08 and CT09 were synthesized by adapting published protocols (Chen et al., 2002; MacKinnon et al., 2007). Intermediates were characterized by  $^1\text{H}$  and  $^{13}\text{C}$  NMR and mass spectrometry. Final compounds were characterized by  $^1\text{H}$  NMR and high-resolution mass spectrometry and found to be in agreement with published data.

### Secretory Protein Profiling

Primary human endothelial, peripheral blood mononuclear, or bronchial epithelial cells were stimulated under various proinflammatory conditions (see Supplemental Experimental Procedures for details) in the presence of increasing concentrations of CT08 or CT09. After 24 hours, protein expression levels were quantified by cellular immunoassays as previously described (Berg et al., 2010). Briefly, microtiter plates containing treated and stimulated cells were blocked and then incubated with primary antibodies for 1 hr. After washing, plates were incubated with a biotin-conjugated anti-mouse IgG antibody for 1 hr, followed by HRP-streptavidin for 30 min. Plates were washed and developed with TMB substrate. Using a SpectraMAX 190 plate reader (Molecular Devices), we read the absorbance at 450 nm. Mean values were calculated from triplicate samples ( $\pm\text{SEM}$ ) and expressed as a normalized ratio versus the DMSO control values. Normalized expression values were plotted as a function of CT08 and CT09 concentration. EC<sub>50</sub> values (with 95% confidence intervals) were estimated by curve fitting with GraphPad Prism software.

### Protein Expression in COS-7 and HeLa Cells

The plasmid encoding human TNF- $\alpha$  was obtained from the PlasmID Repository at Harvard Medical School (clone 1319). The coding region was cloned into pcDNA3.1 (Invitrogen) and used in transient transfection experiments. COS-7 cells were cultured in high glucose Dulbecco's modified eagle medium (DMEM) with 10% FBS at 10% CO<sub>2</sub>. Cells were seeded in 6-well dishes, grown to 80% confluence, and transfected with the TNF- $\alpha$  expression plasmid using Lipofectamine 2000. Five hours after transfection, the media was changed to include CT08 at the indicated concentrations. After 24 hr, cells were harvested in lysis buffer (0.5% Triton X-100 in PBS). Lysates were normalized using the Bradford assay and equal amounts of total protein were resolved by SDS-PAGE and analyzed by immunoblotting and chemiluminescence. Transfection experiments with GFP (Clontech), Prl-GFPKDEL and VCAM-GFPKDEL (both in pDNA3.1) were performed similarly except that HeLa cells growing in 24-well dishes were used. Two hours after transfection, cells were treated with the indicated concentrations of CT08 and CT09 for 20 hr and then lysed in 200  $\mu\text{l}$  1% SDS, 0.1 M Tris (pH 8.0). After boiling and shearing the DNA, aliquots were analyzed by SDS-PAGE and the GFP visualized by western blotting.

### In Vitro Cotranslational Translocation Assays

In vitro transcription, translation, translocation, and protease protection assays were performed as previously described (Sharma et al., 2010). Briefly, DNA templates were transcribed for 60 min at 37°C. Translation reactions

were performed at 32°C for 30 min. Samples were separated by SDS-PAGE, dried, and exposed to Kodak Biomax MR film. For quantitative analysis of translocation experiments, dried gels were exposed to a phosphorimaging screen and quantified using a Typhoon 9400 phosphorimager (Amersham) with accompanying software.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.06.015.

## ACKNOWLEDGMENTS

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**Supplemental Information**

**Secretory Protein Profiling Reveals**

**TNF- $\alpha$  Inactivation by Selective**

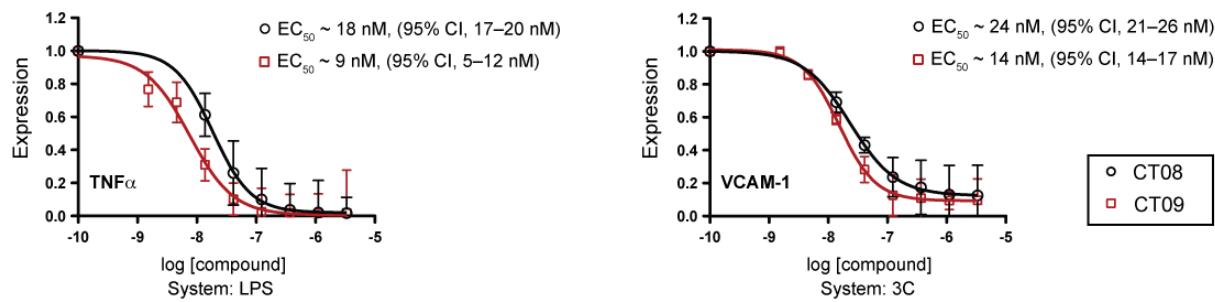
**and Promiscuous Sec61 Modulators**

**Sarah V. Maifeld, Andrew L. MacKinnon, Jennifer L. Garrison, Ajay Sharma, Eric J. Kunkel, Ramanujan S. Hegde, and Jack Taunton**

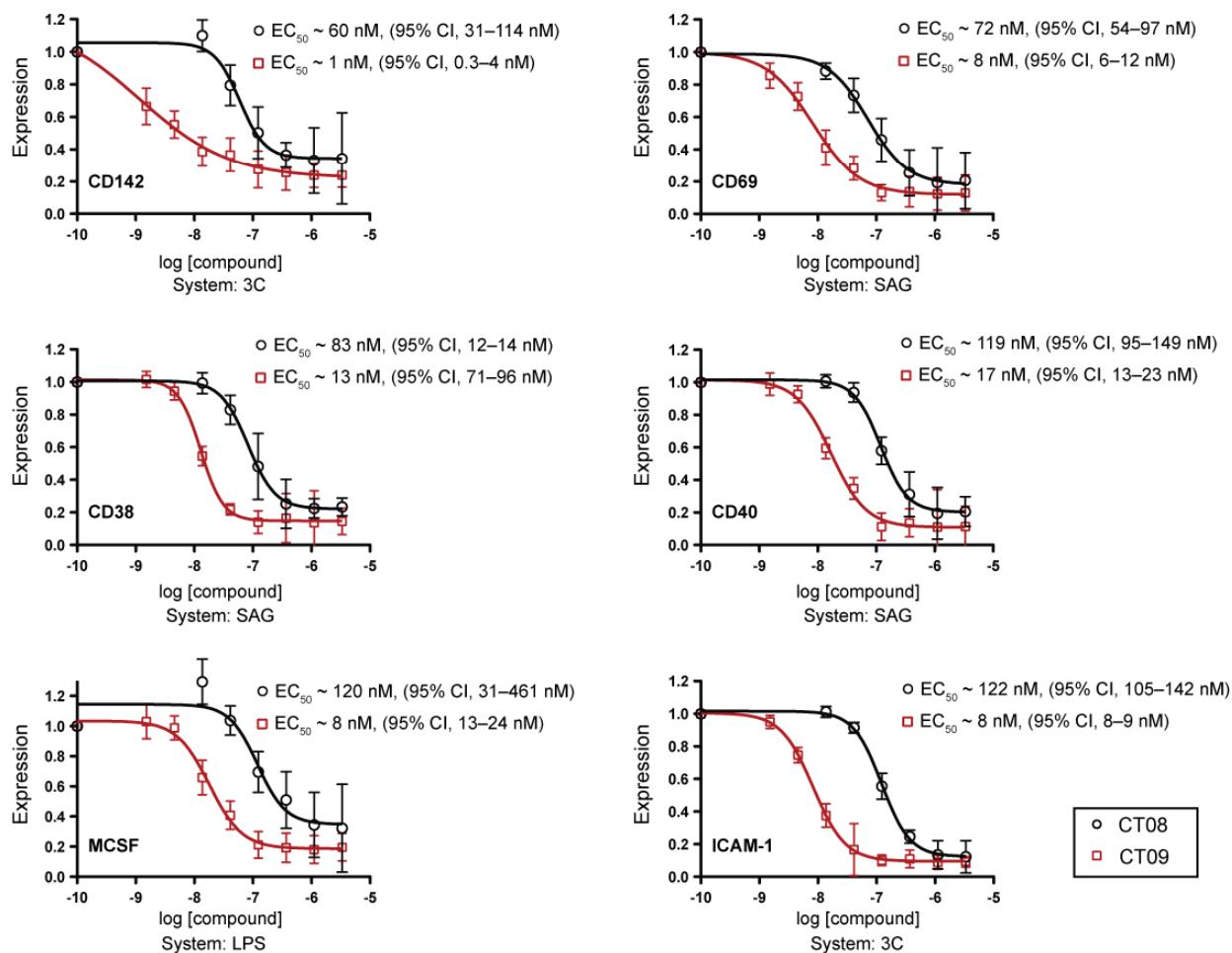
## Figure S1, related to Figure 2. Secretory protein profiling using CT08 and CT09.

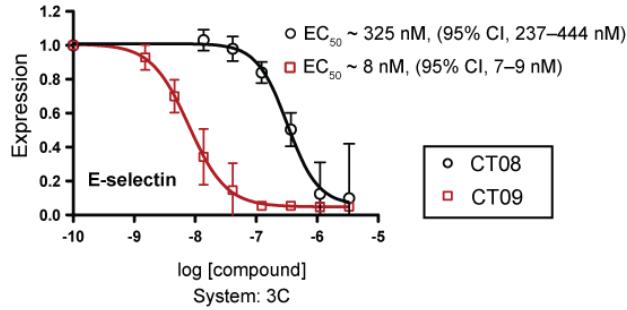
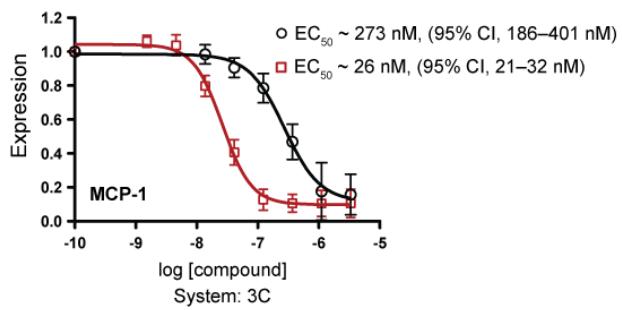
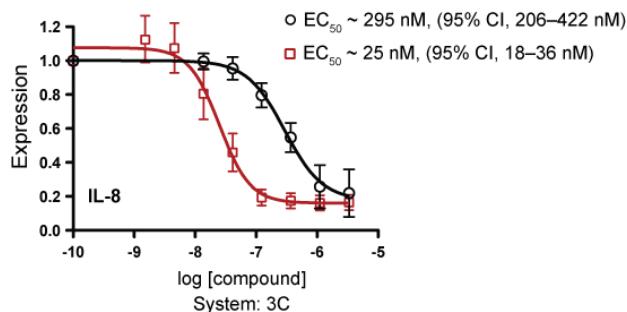
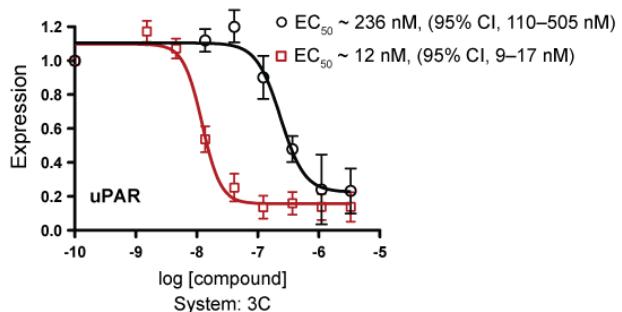
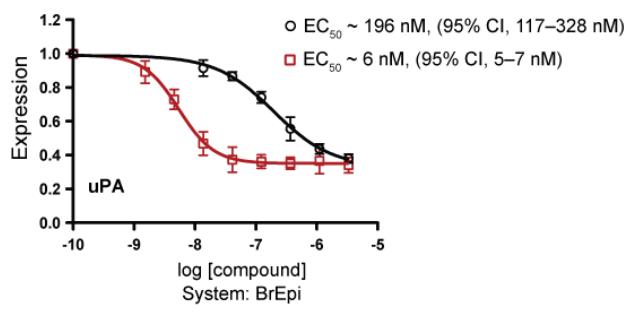
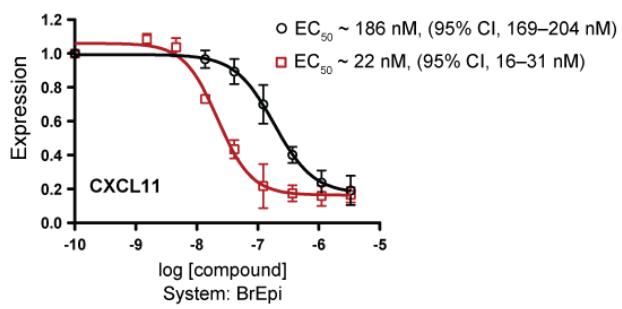
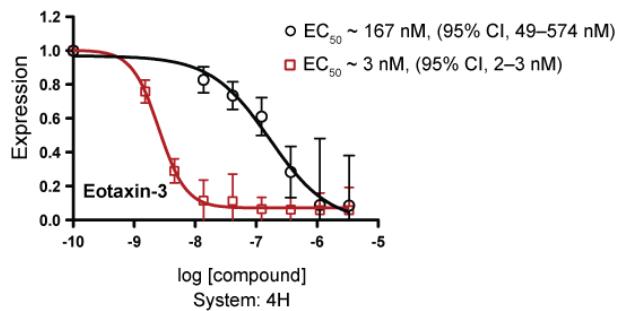
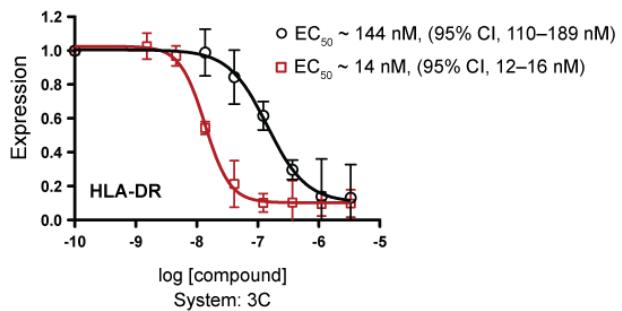
A detailed description of each cell/stimulation system (3C, BrEpi, LPS, 4H, SAG) can be found in the Experimental Procedures.

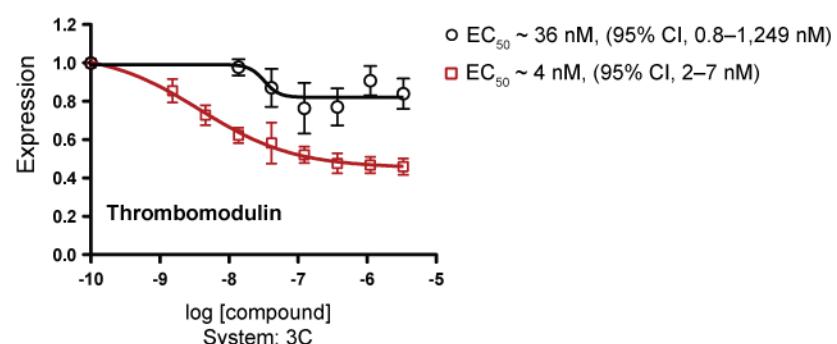
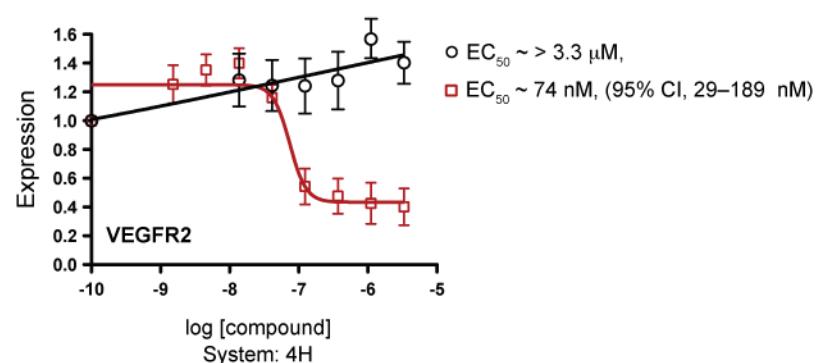
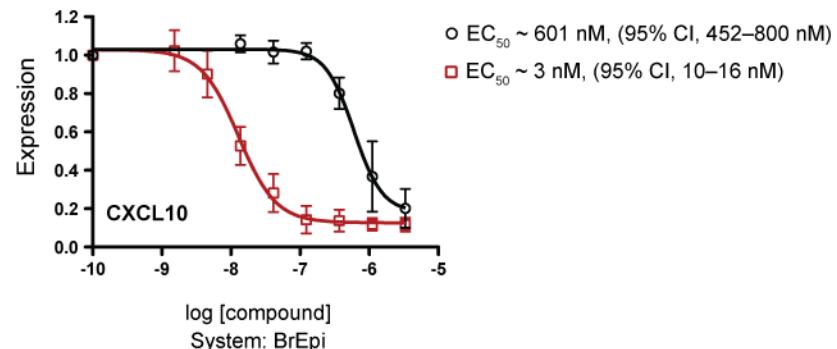
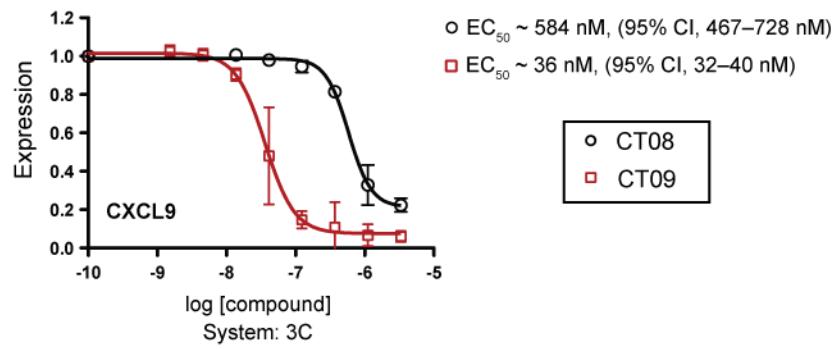
**(A) Class I proteins.** The expression of class I proteins is similarly inhibited by both CT08 and CT09.



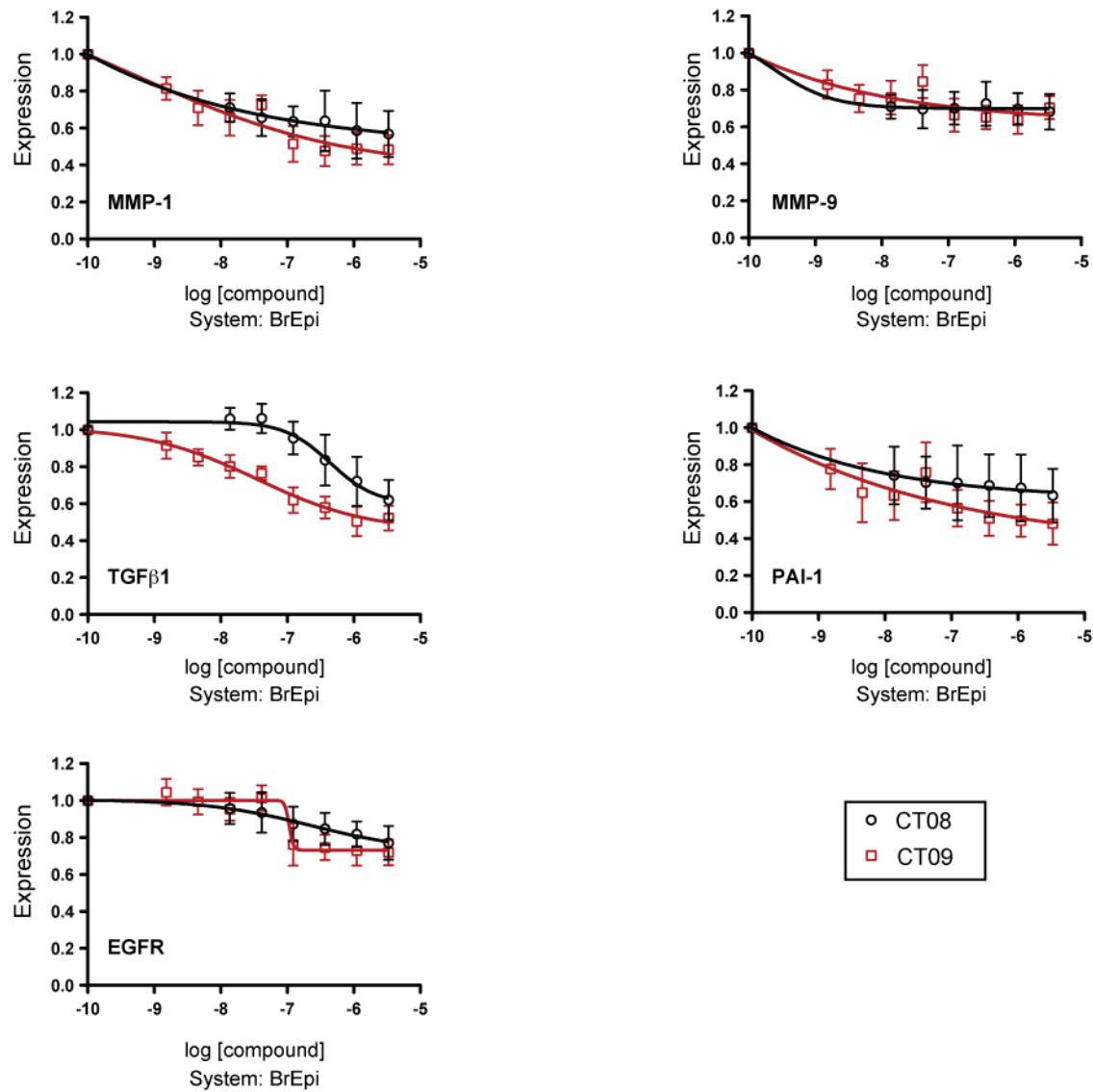
**(B) Class II proteins.** Class II proteins show greater sensitivity to CT09 than CT08.



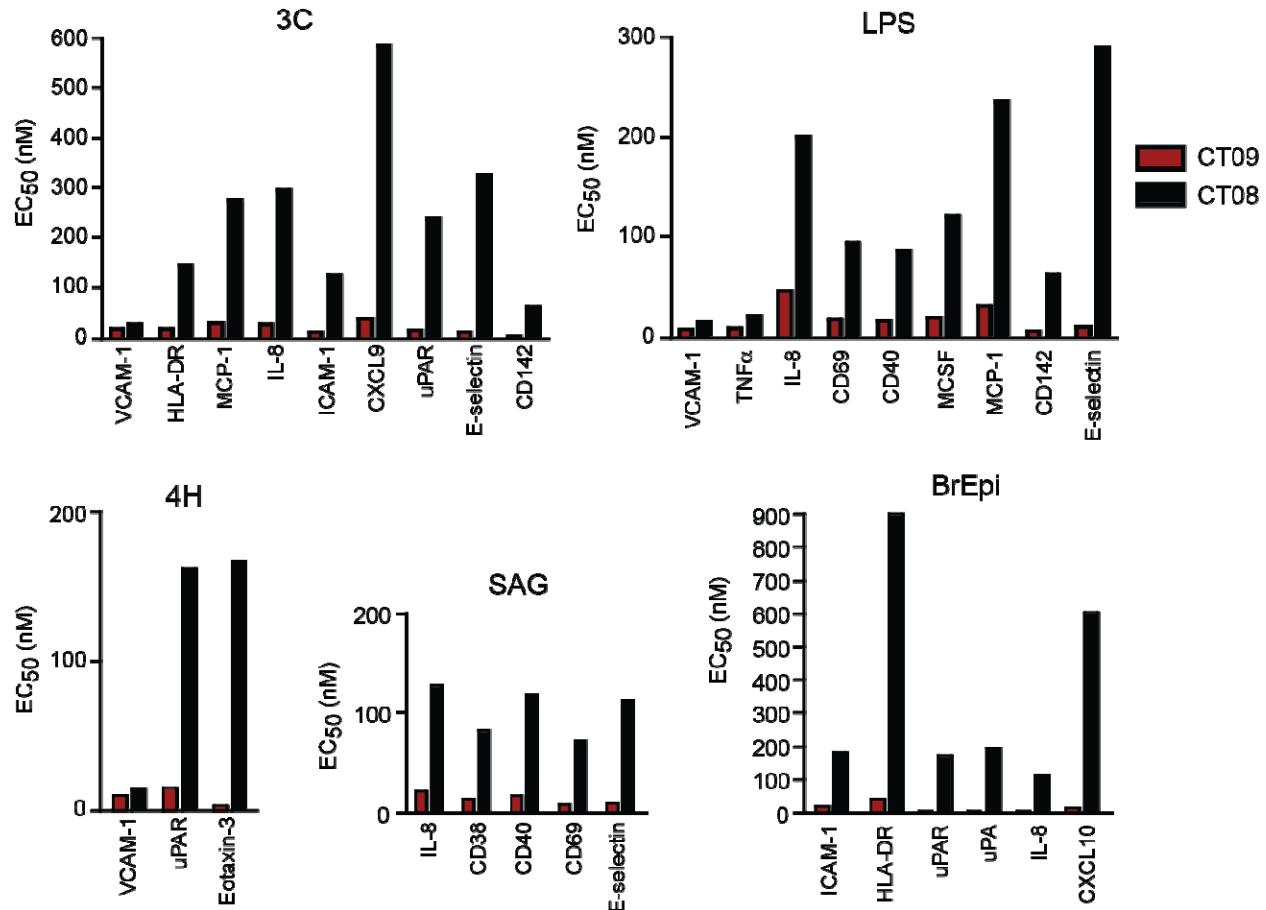




**(C) Class III proteins.** Class III proteins show < 60% inhibition when treated with 3.3  $\mu$ M CT08 or CT09.



**(D) EC<sub>50</sub> values grouped according to the cell system in which they were determined.** Note that the expression of several proteins (e.g., VCAM, ICAM, IL-8) was measured in more than one system. Although the EC<sub>50</sub> values vary somewhat from system to system, VCAM is similarly sensitive to CT08 and CT09 in every system. All other proteins (except TNF $\alpha$ ) were significantly more sensitive (> 2-fold) to CT09 than CT08, regardless of the system.



**Table S1, related to Figure 2.** Proteins assayed by secretory protein profiling.

Protein	UniProt Accession	Classification	N-terminal signal sequence / signal anchor
Eotaxin-3/CCL26	Q9Y258	Secreted	AA 1 – 23: MMGLSLASAVLLASLLSLHLGTA
IL-8/CXCL8	P10145	Secreted	AA 1 – 20: MTSKLAVALLAFLISAALC
CXCL10/IP-10	P02778	Secreted	AA 1 – 21: MNQTAILICCLIFLTLSGIQG
CXCL11/ IP-9	O14625	Secreted	AA 1 – 21: MSVKGMAIALAVILCATVVQG
MCP-1/CCL2	P13500	Secreted	AA 1 – 23: MKVSAALLCLLLIAATFIPQGLA
MCSF	P09603	Secreted	AA 1 – 32: MTAPGAAGRCPPPTWLGSLLLVCLLASRSIT
CXCL9/MIG	Q07325	Secreted	AA 1 – 22: MKKSGVLFLLGIILVLIGVQG
MMP-1	P03956	Secreted	AA 1- 19: MHSFPPLLLLLFWGVVSHS
MMP-9	P14780	Secreted	AA 1 – 19: MSLWQPLVLVLLVLGCCFA
PAI-1	P05121	Secreted	AA 1 – 23: MQMSPALTCLVGLALVFGEGSA
TGF $\beta$ 1	P01137	Secreted	AA 1 – 29: MPPSGLRLLLLLPLLWLLVLTPGRPAAG
uPA	P00749	Secreted	AA 1 – 20: MRALLARLLLGVVVSDSKG
CD40/TNR5	P25942	Type I membrane protein	AA 1 – 20: MVRPLQCVLWGCLLTAVHP
CD142/Tissue Factor	P13726	Type I membrane protein	AA: 1 – 32: METPAWPRVPRPETAVARTLLLGVFAQVAGA
EGFR	P00533	Type I membrane protein	AA 1 – 24: MRPSGTAGAALLALLAALCPASRA
ICAM-1	P05362	Type I membrane protein	AA: 1 – 27: MAPSSPRPALPALLVLLGALFPGPNGA
E-selectin	P16581	Type I membrane protein	AA: 1 – 21: MIASQFLSALTIVLLIKESGA
Thrombomodulin	P07204	Type I membrane protein	AA 1 – 18: MLGVVLGALALAGLGFP
VCAM-1	P19320	Type I membrane protein	AA 1 – 24: MPGKMWVILGASNILWIMFAASQA
VEGFR-2	P35968	Type I membrane protein	AA 1 – 19: MQSKVLLAVALWLCVETRA
CD38	P28907	Type II membrane protein	AA 22 – 42: AQLCLGVSVLVLVVVLAVV
CD69	Q07108	Type II membrane protein	AA 41 – 61: VLCAVMNVVFITILHALIAL
HLA-DR	P04233	Type II membrane protein	AA 47 – 72: GALYTGFISILVTLLLAGQATTAYFLY
TNF $\alpha$	P01375	Type II membrane protein	AA 29 – 51: RCLFLSFLIVAGATTLFCLL
uPAR / CD87	Q03405	GPI-linked	AA: 1 – 22: MGHPPLPLLLLHTCVPASWG

## **Supplemental Experimental Procedures**

### **Biological reagents**

Recombinant human IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$  and IL-4 were obtained from R&D Systems. Staphylococcal enterotoxin B (SEB), toxic shock syndrome toxin-1 (TSST; Staphylococcal enterotoxin F) from *S. aureus* (collectively called superantigen; SAG), lipopolysaccharide from *Salmonella enteritidis*, histamine and proteinase K (PK) were purchased from Sigma-Aldrich. For  $^{35}$ S-incorporation during translation experiments, Tran $^{35}$ S-Label was purchased from MP Biomedicals. Biotin-conjugated anti-mouse IgG antibody was purchased from Cedarlane Laboratories; HRP-streptavidin was purchased from Jackson Labs. TMB (3,3,5,5-tetramethylbenzidine) substrate was purchased from Clinical Sciences Products, Inc. Lipofectamine 2000 was purchased from Invitrogen.

For the cellular immunoassays, mouse antibodies against human proteins were purchased from commercial sources: VEGFR2 (Sigma); TNF $\alpha$  (Invitrogen); tPA (Upstate); CD142 (Calbiochem); ICAM-1 (Beckman Coulter); E-selectin (Hycult Biotechnology); VCAM-1, HLA-DR, CD40, CD69, CXCL9, MCP-1, thrombomodulin, EGFR, uPAR and CD38 (BD Biosciences); Eotaxin-3, CXCL10, CXCL11, IL-8, MMP-1, MMP-9, TGF- $\beta$ 1 and M-CSF (R&D Systems); PAI-1 (Oncogene/EMD); uPA (Millipore).

Mouse monoclonal antibodies for Western blot detection of human TNF $\alpha$  and actin were purchased from R&D Systems and Sigma-Aldrich, respectively. HRP-conjugated goat anti-mouse secondary antibody was purchased from Santa Cruz Biotechnology. ECL reagent for chemiluminescence was purchased from GE Healthcare.

### **Secretory protein profiling**

Human umbilical vein endothelial cells (HUVECs) were obtained from Cascade Biologics and cultured as previously described (Plavec, et al., 2004). Primary human bronchial epithelial cells (Cell Applications, Inc.) were cultured according to methods recommended by the manufacturer. Peripheral blood mononuclear cells (PBMCs) were prepared from buffy coats (Stanford Blood Bank, Stanford, CA) by centrifugation over Histopaque-1077 (Sigma). Cells were cultured to confluence in microtiter plates. Compounds dissolved in DMSO (final concentration 0.1–0.2%) were added to cells 1 h before stimulation and were present during the entire 24 h stimulation

period. CT08 was tested from 0.012–3.3  $\mu$ M. CT09 was tested down to 0.001  $\mu$ M, as preliminary experiments indicated that it was significantly more potent than CT08 toward most proteins. Five cell/stimulation systems (3C, BrEpi, 4H, LPS, SAG) were employed to measure the expression of 25 secreted and transmembrane proteins. The system used for each secreted/transmembrane protein, along with each dose-response curve, is provided in Fig. S1 and S2. For the 3C and BrEpi systems, HUVECs or bronchial epithelial cells, respectively, were stimulated with IL-1 $\beta$ , (1 ng/ml), TNF $\alpha$  (5 ng/ml) and IFN- $\gamma$  (20 ng/ml). For the 4H system, HUVECs were stimulated with IL-4 (5 ng/ml) and histamine (10  $\mu$ M). For the LPS system, HUVECs and PBMCs were stimulated with lipopolysaccharide (2 ng/ml). For the SAG system, HUVECs and PBMCs were stimulated with SAG (20 ng/ml).